Yeast src Homology Region 3 Domain-binding Proteins Involved in Bud Formation

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Abstract. The yeast protein Bemlp, which bears two src homology region 3 (SH3) domains, is involved in cell polarization. A Rho-type GTPase, Rho3p, is involved in the maintenance of cell polarity for bud formation, and the rho3 defect is suppressed by a high dose of BEM1. Mutational analysis revealed that the second SH3 domain from the NH2 terminus (SH3-2) of Bemlp is important for the functions of Bemlp in bud formation and in the suppression of the rho3 defect. Boi2p, which bound to SH3-2 of Bemlp, was identified using the two-hybrid system. Boi2p has a proline-rich sequence that is critical for displaying the Boi2p-Bemlp two-hybrid interaction, an SH3 domain in its COOH-terminal half, and a pleckstrin homology domain in its NH2-terminal half. A BOI2 homologue, BOII, was identified as a gene whose overexpression inhibited cell growth. Cells overexpressing either BOII or BOI2 were arrested as large, round, and unbudded cells, indicating that the Boi proteins affect cell polarization. Genetic analysis revealed that BOII and BOI2 are functionally redundant and important for cell growth. Aboi2 cells became large round cells or lysed with buds, displaying defects in bud formation and in the maintenance of cell polarity. Analysis using several truncated versions of BOI2 revealed that the COOH-terminal half, which contains the pleckstrin homology domain, is essential for the function of Boi2p in cell growth, while the NH2-terminal half is not, and the NH2-terminal half might be required for modulating the function of Bemlp. Overproduction of either Rho3p or the Rho3p-related GTPase Rho4p suppressed the boi defect. These results demonstrate that Rho3p GTPases and Boi proteins function in the maintenance of cell polarity for bud formation.

During bud formation in the yeast Saccharomyces cerevisiae, cell polarity is established for the initiation of bud emergence and it is maintained during bud growth. Patches of actin filaments become concentrated at the bud site, towards which the transport of secretory vesicles is directed for the construction of the daughter cell (Tkacz and Lamphen, 1972; Field and Schekman, 1980; Pringle and Hartwell, 1981; Cabib et al., 1982; Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Pringle et al., 1986; Drubin, 1991). The establishment and maintenance of cell polarity require the functions of Rho-type GTPases Cdc42p, Rhop, and Rho4p, which belong to the Ras superfamily (Johnson and Pringle, 1990; Matsui and Toh-e, 1992a, b; Imai et al., 1996). GTPases of the Ras superfamily act as molecular switches through their conformational change between the GTP-bound active form and GDP-bound inactive form (Barbacid, 1987; Bourne et al., 1991; Boguski and McCormick, 1993). Defects in either CDC42 or CDC24, which encodes a GTP-GDP exchange factor for Cdc42p, disrupt the asymmetric localization of actin filaments and cause cells to become unbudded, large, and round, an indication that Cdc42p and Cdc24p are essential for the establishment of cell polarity (Sloat and Pringle, 1978; Sloat et al., 1981; Adams et al., 1990; Johnson and Pringle, 1990; Zheng et al., 1994).

Defects in RHO3 cause severe growth defects. Disruption of RHO4, which encodes a Rho3p-related GTPase, enhances the growth defect of Δrho3 cells (Matsui and Toh-e, 1992a). Temperature-sensitive rho3 mutant cells lose cell polarity at nonpermissive temperatures; the asymmetric localization of actin filaments is disrupted in the rho3 cells, and the rho3 cells are arrested as large, round cells, although, in contrast to cdc42 mutant cells, not all of these cells are arrested as unbudded cells (Imai et al., 1996). Depletion of both Rho3p and Rho4p results in lysis of cells that have small buds (Matsui and Toh-e, 1992b). These observations strongly suggest that Rho3p is required for the maintenance of cell polarity for bud growth.

The rho3 defect is suppressed by the overexpression of BEM1, an indication that Bemlp has functions that affect...
the rho3 pathway (Matsui and Toh-e, 1992b). BEM1 encodes a protein with two copies of the src homology region 3 (SH3) domain (SH3-1 and SH3-2; see Fig. 1 A). Disruption of BEM1 results in temperature-sensitive growth. At nonpermissive temperatures, abem1 cells become unbudded, large, and round, with the loss of actin polarization, indicating that Bem1p is involved in cell polarization (Bender and Pringle, 1991; Chenever et al., 1992). Bem1p can bind to Cdc24p independently of its SH3 domains (Peterson et al., 1994). Cdc24p binds to the Ras-type GTPase Rsr1p/Bud1p, which is required for the proper selection of bud sites (Chant and Herskowitz, 1991; Bender and Pringle, 1989; Zheng et al., 1995). These findings lead to the possibility that Bem1p is a component of the protein complex that is needed for bud emergence. Since SH3 domains mediate protein–protein interactions (e.g., Cicchetti et al., 1992), it is possible that Bem1p recruits another component to the complex via its SH3 domains.

To clarify the role of the SH3 domains of Bem1p, we characterized several bem1 mutants that were defective in these domains. Using the two-hybrid system, we identified Boi2p as a protein that bound to the SH3 domain and was important for the function of Bem1p. In addition, Boi1p, which was functionally redundant with Boi2p, was identified by its inhibitory effect on bud emergence. Our genetic and morphological studies indicate that the function of Boi protein is related to the Rho3 pathway and is important for bud growth. We present a model in which Boi proteins and Rho3p are involved in the modulation of the Bem1p-containing complex for bud growth.

Materials and Methods

Microbiological Techniques

Rich medium containing glucose (YPD), synthetic minimal medium (SD), and synthetic complete medium (SC) are as described (Sherman et al., 1986). YPGal and SCGal are YPD and SC, respectively, except that 2% glucose is replaced with 5% galactose and 0.3% sucrose. YPGal0.1 is YPGal, except that the concentration of galactose is 0.1%. SC-U and SCGal-U are SC and SCGal, respectively, without uracil. SC-L and SCGal-L are SC and SCGal, respectively, without leucine. SC-UT and SCGal-UT are SC and SCGal, respectively, without uracil and tryptophan. Yeast transformations were performed by the method of Ito et al. (1983).

Strains and Plasmids

The yeast strains used are listed in Table I. Plasmid pBTM116 is a high copy number plasmid that harbors the 2-μm DNA origin TRP1 and the sequence for the lexA DNA-binding domain. pGAD424 is a high copy number plasmid that harbors the 2-μm DNA origin LEU2 and the sequence for the Gal4 trans-activation (Gal4 TA) domain (Fields and Sternglanz, 1994). Plasmid pRS316-RH03 carries a 1.8-kb KpnI-XhoI fragment that encompasses the Rh03 gene on pRS316, a low copy number plasmid that carries the 2-μm DNA origin (Sikorski and Hieter, 1989). Plasmid pKT10 is a high copy number plasmid harboring URA3 (Sikorski and Hieter, 1989). Plasmid pKT10 is a high copy number plasmid that harbors the 2-μm DNA origin URA3, the TDH3 promoter before a unique EcoRI site, termination codons downstream of the EcoRI site, and the TDH3 terminator (Tanaaka et al., 1988). pOPR3 and pOPR1 are pKT10-based plasmids that carry the coding region of RH03 and the coding region of RHO1, respectively, under the control of the TDH3 promoter (Matsui and Toh-e, 1992b). Plasmid pKT10myc was constructed by inserting the sequence for the initiator methionine, a myc epitope tag (EQQKLISEEDL), and a multicloning site into the EcoRI site of pKT10. The Kpn1-Kpn1, BamHI-Kpn1, HpaI-Kpn1, and Kpn1-Dral fragment carrying the BEM1 sequence (see Fig. 1 A) were inserted in frame into the multicloning site of pKT10myc to generate pBEM1KK, pBEM1A1, pBEM1ΔΔ2, and pBEM1ΔΔC, respectively. The 1.1-kb EcoRI-SalI fragment carrying the sequence for SH3-2 was removed from pBEM1KK and pBEM1ΔΔC to create pBEM1ΔΔ2 and pBEM1ΔΔΔC, respectively, and was religated in frame after blunting the overhangs. BEM1 in pBEM1KK lacked the sequence for the 45 NH2-terminal amino acids but was able to serve as a multicycop suppressor of rho3 (see Fig. 1 B, sector 2). The DNA sequences of the fragment, which were derived from the PCR (Saito et al., 1988) or oligonucleotide-directed mutagenesis and used for generating plasmids, were determined to confirm precise replication during each procedure. Nucleotide sequences were determined by the method of Sanger et al. (1977).

Construction of bem1 Mutants with Mutations in the SH3 Domain

The 2.3-kb fragment, derived from pSRO1 (Matsui and Toh-e, 1992b), from the BamHI site in the BEM1 coding region to the BamHI site in the 3′ noncoding region, was inserted into a derivative of pBluescriptII that had been constructed from pBluescriptII KS+ (Strategene, La Jolla, CA) by removal of the sequence between the EcoRV and XhoI sites. The 1.1-kb HindIII-HindIII fragment carrying URA3 was inserted into the HindIII site (in the 3′ noncoding region of BEM1) of the resultant plasmid to generate YipUBEM1C. The 0.9-kb fragment, derived from the SalI site in the Smal site in the 5′-coding region of BEM1 to the YipUBEM1C in the sequence for SH3-2 of YipUBEM1C to generate YipUBEM1. Proline 123 in SH3-1 and proline 208 in SH3-2 were replaced with leucine to generate bem1leu123 and bem1leu208, respectively, with an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp., Arlington Heights, IL) using the primers 5′-AAAATAGGTCTTAAGGACAACACC (for bem1leu123), 5′-AAAAACCACTGATCAAGGCGCG (for bem1leu208), and YipUBEM1 as the template. For the construction of bem1ASH3s, a truncated version of bem1 without the sequence for both SH3-1 and SH3-2, the fragment from the Smal site in the 5′ noncoding region of the Kpn1 site in the coding region was inserted in frame between the Smal and YipUBEM1C after blunting the overhangs to generate YipUBEM1ASH3s, which lacked the sequence between the Kpn1 and SalI sites of YipUBEM1. For the construction of bem1AΔC, the 1.0-kb XhoI-HindIII fragment from pBEM1ΔΔC, which contained the BEM1 sequence between the SalI and DraI sites, the termination codons after the DraI cleavage site, and the TDH3 terminator, was inserted between the SalI and HpaI sites of YipUBEM1 after blunting the overhangs of the HindIII cleavage site for the replacement of the wild-type BEM1 allele with a mutant allele, each derivative of YipUBEM1, digested with Smal and XbaI, was introduced into the cells. Replacement was confirmed by PCR.

Isolation of BOI2 and Assay of the Two-Hybrid Interaction

The construction of plasmids for the lexA DNA-binding domain fused with Bem1p (lexA-Bem1p) was performed as follows. An EcoRI site was introduced before the initiator methionine codon of BEM1 by oligonucleotide-directed in vitro mutagenesis. The EcoRI-HpaI fragment of BEM1 was inserted into the multicloning site of pBTM116 in frame to generate plexSHs. The SacI-SalI fragment that contained the sequence for the COOH-terminal Bem1p and the 3′ noncoding region of BEM1 from pBEM1KK was inserted between the SalI sites of plexSHs to create plexBEM1. Yeast strain L40, with two reporter genes (lexA-lacZ and lexA-HIS3), was transformed with plexBEM1 and then with a yeast genomic library in which yeast genomic DNA was expressed as fusion proteins with the Gal4 TA domain (Chien et al., 1991). The transformants were streaked on SD+3AT plates, which are SD plates containing 100 μg/ml adenine sulfate and 40 mM 3-aminotriazole, an inhibitor of imidazoleglycerolphosphate dehydratase (Igp3p). Plasmids were recovered from colonies that formed on plates after incubation at 25°C for 1 wk. The recovered plasmids were reintroduced into the L40 strain harboring plexBEM1 to examine whether the plasmids could induce the expression of the reporter genes. HIS3 expression was assessed by the formation of L40 cell colonies on SD+3AT plates after incubation at 25°C for 1 wk.

1. Abbreviations used in this paper: a.a., amino acid; GST, glutathione S-transferase; FH, pleckstrin homology; SC, synthetic complete medium; SD, synthetic minimal medium; SH3, src homology region 3; YPD, rich medium containing glucose.
lacZ expression was examined by measuring the activity of β-galactosidase in L40 cells with the method described (Miller, 1972). DNA clones carrying BOI2 were isolated from a yeast genomic library (Matsui and Toh-e, 1992a) in XZAPII (Stratagene) by hybridization using the plasmid derived from the yeast genomic library and carried a 6-kb fragment encompassing BOI2 in pBluescript SK- (Stratagene).

Construction of Plasmids for the Two-hybrid Interaction Assay

Plasmids for truncated versions of lexA-Bemlp were constructed as follows. pLexSHs was created by removing the EcoRI-Sall fragment of pLexBEM1, plexAC for SH3-2) fragment of plexBEM1, with partial digestion by SalI. plexAC and plexAC were constructed by inserting BanI-PstI fragments from pBEM1AC was inserted between the Sall site of pGADR1 to create pGADAPRO. The 1.8-kb EcoRI-BamHI fragment carrying the sequence for the COOH-terminal half of Cdc24p (in frame to generate pSKBOI2APRO. The mutagenesis, and the resultant plasmid was digested with AatlI and religated in frame to generate pSKBOI2APRO. The mutagenesis, and the resultant plasmid was digested with AatlI and religated

In Vitro Binding Assay

The NciI-Ecorl fragment encoding Boi2p (amino acids [a.a.] 67–545) was inserted into the multi-cloning site downstream of the sequence for glutathione S-transferase (GST) in pGEXKG. Boi2p (a.a. 67–545) fused with GST (GST-Boi2p) was produced by use of this construct and affinity purified with glutathione-agarose beads (Sigma Immunoenzymes, St. Louis, MO) as described (Shirayama et al., 1994). About 10⁸ yeast cells (wild-type strain KA31) producing myc-tagged Bem1p were washed twice in PBS. The cell pellet was disrupted by blending with glass beads in 400 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2% (vol/vol) Triton X-100, 0.5 mM PMSF) and was clarified by centrifugation at 14,000 g for 15 min at 4°C. 30 μl of the supernatant (as the total cell lysate) was resolved by SDS-PAGE, and 160 μl of the supernatant was mixed with 50 μl GSH-agarose beads with 2 μg immobilized GST-Boi2p (or GST) and incubated at 4°C for 3 h. The beads were then washed extensively in washing buffer (50 mM Tris-HCl, pH 7.5, 0.1% [vol/vol] Triton X-100, 10% [vol/vol] glycerol). Bound proteins were eluted with 100 μl elution buffer (50 mM Tris-HCl, pH 9.0, 20 mM glutathione) and 20 μl of the eluate were resolved by SDS-PAGE.

Isolation of BOI1

YEp51B, which carries LEU2, the 2-μm DNA origin, and the GAL10 promoter, was constructed by removing a 0.27-kb BamHI-Sall fragment adjacent to the GAL10 promoter from YEp51 (Broach et al., 1983). Diploid strain DRA4 was transformed with a yeast genomic DNA library, based on YEp51B, in which yeast genomic DNAs were expressed under the control of the GAL10 promoter. The resultant transformants that grew well on SC-L, but not on SCGal-L (which contained 2% galactose, instead of 5% galactose and 0.3% sucrose), were selected at 28°C, and the plasmids were recovered (details of the screening will be described elsewhere). pGA68, one of the isolates, carried BOI1 downstream of the GAL10 promoter. The BOI1 gene in pGA68 was expressed from the internal methionine at position 378, as judged from a comparison of the nucleotide sequences of pGA68 and BOI1 (Bender et al., 1996).

BOI2 Expression Plasmids and Disruption of BOI1 and BOI2

YtpUGAL7 is a Ytp plasmid that carries the GAL7 promoter. URA3, and the BglIII and Sall sites downstream of the GAL7 promoter (Matsui and Toh-e, 1992b). Plasmids for truncated versions of lexA-Bemlp were constructed as follows. MluI-BglII, MluI-EcoRI, and MluI-Sall sites of pGAD24 were replaced with AatlI and religated in frame to generate pGADAPRO. The 1.8-kb EcoRI-BamHI fragment carrying the sequence for COOH-terminal half of Cdc24p (a.a. 167-end) was inserted between the EcoRI and BamHI sites of pGAD24 to create pGADDC24.
Toh-e, 1992b). A BamHI site was introduced before the initiator methionine codon of $BOI_2$ by oligonucleotide-directed in vitro mutagenesis, and the BgIII site in the 3' noncoding region of $BOI_2$ was replaced with a SalI site by insertion of a SalI linker (Takara, Tokyo, Japan). The BamHI-BglII fragment carrying the complete $BOI_2$ coding region was inserted between the BgIII and SalI sites of YipUgAL7 to create YipUgAL7BOI2. In this construction, the pGAL7:BOI2AC, $BOI_2$ was expressed under the control of the GAL7 promoter. YipUgAL7BOI2 was digested with BgIII and religated after blunting the overhangs to create YipUgAL7BOI2AC. From this construct, designated pGAL7:BOI2AC, $Boi_2$ was digested with BglII and religated after blunting the overhangs to create pYO324-BOI2AC and pRS314-BOI2AC, respectively. From these constructs, designated YipUgAL7BOI2AN and YipUgAL7BOI2AN, $Boi_2$ without its NH2-terminal half was produced under the control of the GAL7 promoter. An ~6-kb SacII-SalI fragment carrying $Boi_2$ from pSKBOI2 was inserted into pYO324 and a high copy number plasmid that carries 2-μm DNA origin and TRP1 (Ohya et al., 1991), and into pRS314, a low copy number plasmid that carries CEN6 and TRP1 (Sikorski and Hieter, 1989), to create pYO324-BOI2 and pRS314-BOI2, respectively. An AatII site of pSKBOI2 was introduced downstream of the 9th codon of $BOI_2$ in pSKBOI2 by oligonucleotide-directed in vitro mutagenesis, and the resulting plasmid was digested with AatII and religated in frame to create pSKBOI2AN. This construct (pSKBOI2AN) encodes $Boi_2$ without its NH2-terminal half (a.a. 10-464). An ~5-kb SacII-SalI fragment carrying YipUgAL7BOI2AN was inserted into pRS306, a Yip plasmid carrying URA3 (Sikorski and Hieter, 1989), to create pYO324-BOI2AN and pRS306-BOI2AN, respectively. pYO324-BOI2 and pRS314-BOI2 were digested with BgIII and religated after blunting the overhangs to create pYO324-BOI2AC and pRS314-BOI2AC, respectively. From these constructs, designated YipUgAL7BOI2AN, $Boi_2$ without its COOH-terminal half (a.a. 697-end) was produced. The YipUgAL7- and pRS306-derived plasmids were digested with Stul and then introduced into cells for target integration at the ura3 locus. The $BOI_2$ sequence between the Pmtll site (in the 5' noncoding region) and the SpeI site of pSKBOI2 was replaced with the 1.1-kb URA3 fragment to create pBOI2A. In this construct, the sequence from the position 443 bp upstream of the initiation codon to the 829th codon of $BOI_2$ was deleted. The plasmid for the disruption of $BOI_1$ was constructed as follows. The 2.8-kb Sall-Hindlll fragment from the insert of pGAl68 was inserted into pBluecriptII K5* to create pSKBOI1. To create pRS306-BOI1, the 2.2-kb BamHI-BamHI fragment that carried the 3'-half of the $BOI_1$ gene from pGAl68 was inserted into the BamHI site of pRS306. To clone the 5'-half of $BOI_1$, the yeast genomic DNA in which pRS306-BOI1 had been integrated at the $BOI_1$ locus was digested with EcoRV, religated, and introduced into Escherichia coli. With the recovered plasmid as a template, a 840-bp fragment carrying the 5' noncoding region of $BOI_1$ and the sequence for the NH2-terminal portion (a.a. 1-87) of $Boi_1p$ was digested with BglII and religated after blunting the overhangs to create pRS306-BOI1AC and pRS314-BOI1AC, respectively. From these constructs, designated YipUgAL7BOI1AN, $Boi_1$ was digested with BglII and religated after blunting the overhangs to create YipUgAL7BOI1AN, indicating that SH3-1 and SH3-2 of $Boi_1$ were deleted. The replacement of the conserved proline residue by leucine destroys the function of an SH3 domain in the ser25 protein (Rose, 1975). A solution of methylene blue (0.02% methylene blue, 2% sodium citrate) was mixed with an equal volume of cell culture, and the cells were observed immediately with Nomarski optics.

Results

Bemlp Domains Required for Suppression of the ARho3 Defect

Overexpression of BEM1 suppresses the growth defect caused by the disruption of RHO3 (Matsui and Toh-e, 1992b; Fig. 1 B, sector 2). To examine whether the SH3 domains of Bemlp are necessary for the suppression or not, we constructed truncated BEM1 sequences (shown diagrammatically in Fig. 1 A) and analyzed the suppression activities of the various constructs. ARho3 cells that overexpressed BEM1 without the sequence for SH3-1 grew as well as ARho3 cells that overexpressed BEM1 (Fig. 1 B, right, sectors 2 and 3), indicating that SH3-1 is not required for the ability of BEM1 to serve as a multicopy suppressor of rho3. However, ARho3 cells that overexpressed BEM1 without the sequence for SH3-2 (Fig. 1 B, right, sectors 4, 5, and 7) or the COOH terminus of 35 a.a. (Fig. 1 B, right, sectors 6 and 7) formed colonies as tiny as those of ARho3 cells that harbored a control plasmid (Fig. 1 B, right, sector 8), indicating that these truncated versions of BEM1 did not suppress the ARho3 defect. The deletion of SH3-2 or of the COOH-terminal sequence did not reduce the amount of the myc-tagged Bemlp, as judged by Western analysis (data not shown). These results indicate that SH3-2 and the COOH-terminal sequence are critical if BEM1 is to function as a multicopy suppressor of rho3.

The Effects of Substitutions in the SH3 Domains and of Deletion of the COOH-terminal Region of Bemlp on Cell Growth

The replacement of the conserved proline residue by leucine destroys the function of an SH3 domain in the sem5 protein of Caenorhabditis elegans (Rozaks-Adcock et al., 1992). We introduced the corresponding mutation into SH3-1 (bemlp Leu23) and SH3-2 (bemlp Leu28). The bemlp Leu23 cells and bemlp Leu28 cells displayed temperature-sensitive growth (Fig. 1 C, sectors 3 and 4). At 37°C, the cells were arrested as large, round, un budded cells, and the asymmetric organization of actin filaments was disrupted in these cells (data not shown). These phenotypes are similar to those of the $Abemlp$ cells. By contrast, bemlp Leu123 cells grew as well as wild-type cells, even at elevated temperatures (Fig. 1 C, sector 2). These results indicate that SH3-2 is important for the function of Bemlp in bud emergence.
The COOH-terminal 35 a.a. of Bemlp is essential for the suppression of the \( \Delta \rho h3 \) defect (Fig. 1 B). We examined whether the COOH-terminal 35 a.a. is also important for the function of Bemlp in cell growth or not. We replaced the wild-type \( BEM1 \) allele with a truncated allele that lacked the sequence for the COOH-terminal 35 a.a. (\( bem1-\Delta C \)). The \( bem1-\Delta C \) cells displayed temperature-sensitive growth (Fig. 1 C, sector 5) and were arrested as large, round, unbudded cells at 37°C (data not shown). These results indicate that the COOH-terminal 35 a.a. is important for the function of Bemlp in bud emergence.

It has been reported that the COOH-terminal half of Bemlp interacts with the COOH-terminal half of Cdc24p (Peterson et al., 1994). We examined whether the COOH-terminal 35 a.a. of Bemlp is required for the Cdc24p-Bemlp interaction using a two-hybrid system (Fields and Sternglanz, 1994). The lexA-Bemlp fusion (from plexBEM1) increased the level of expression of \( \text{lacZ} \) in L40 cells (lexA-lacZ strain), with the increase depending on the presence of the Ga14TA-Cdc24p fusion from pGADCDC24. The lexA-fused Bemlp without the COOH terminus of 35 a.a. (from plexBEM1\( \Delta C \)), however, did not (Table II). These results indicate that the
COOH-terminal 35 a.a. of Bem1p is required for the two-hybrid interaction between Cdc24p and Bem1p.

**Isolation of BOI2**

A screening was made for genes that encode the Bem1p-binding protein using the two-hybrid system. Plasmid plexBEM1, carrying the sequence encoding the lexA-Bem1p fusion and the yeast S. cerevisiae genomic library expressed as fusion proteins with the Gal4 TA domain (Chien et al., 1991), were introduced into L40 cells that carried lexA-lacZ and lexA-HIS3. The plasmids were then recovered from the colonies that formed on selective plates (SD+3AT). Among the plasmids recovered from the His+ transformants, only plasmid pGADBOI2 increased the level of expression of both HIS3 and lacZ in L40 cells in a plexBEM1-dependent manner. DNA clones were isolated from the yeast genomic library (Matsui and Toh-e, 1992a) with a fragment from pGADBOI2 as a probe. The nucleotide sequence of the clones revealed a homologue with 38% identity. This homologous gene was identified on the basis of its two-hybrid interaction with Bem1p (Bender et al., 1996). Both groups designated these genes BOI1 and BOI2 (as bem one interacting), and the gene on pGADBOI2 was designated BOI2. Four domains of Boi2p, namely, domain I (residues 39–113) including an SH3 domain (residues 50–102), domain II (residues 266–331), domain III (residues 436–462) including a proline-rich sequence (residues 438–458), and domain IV (residues 731–943) including a PH domain (residues 767–891), were highly homologous to those of Boi1p (Fig. 2 B), with the extent of identity being 71, 65, 78, and 69%, respectively.

**Domains That Are Required for the Two-hybrid Interaction between Bem1p and Boi2p**

To identify the sequence responsible for the two-hybrid interaction between Bem1p and Boi2p, several constructs that encoded lexA-Bem1p fused proteins with truncation and/or mutations (shown diagrammatically in Fig. 3 A) were introduced into L40 cells that harbored pGADBOI2, and the two-hybrid interaction was examined. The BEM1 sequence for the NH2-terminal half (a.a. 1–325), which contained both SH3 domains, was sufficient for the interaction (Fig. 3 A, plexΔC). Introduction of the bem1Lex123 mutation into this BEM1 sequence, however, abolished the increase in the expression level of the reporter genes (Fig. 3 A, plex-s2 and plex-s12). By contrast, the plasmid carrying only the bem1Lex123 mutation increased the expression (Fig. 3 A, plex-s1). These results indicate that SH3-2 is essential for the Bem1p–Boi2p interaction.

To identify the Boi2p domain that participates in the two-hybrid interaction, we constructed several plasmids that encoded Gal4TA–Boi2p fused proteins, as shown diagrammatically in Fig. 3 B. In the original isolate, pGADBOI2, the BOI2 sequence was ligated with the sequence for Gal4TA at the Sau3AI site that was located in the sequence for the SH3 domain of Boi2p, and the SH3 domain in the fusion was disrupted (see Figs. 2 A and 3 B). Thus, the SH3 domain of Boi2p was dispensable for the Boi2p–Bem1p interaction. The deletion of the COOH-terminal sequence of Boi2p (threonine 465–end) did not reduce the interaction (Fig. 3 B, pGADAA1), an indication that the NH2-terminal half of Boi2p is sufficient for the interaction. More extensive deletion of the COOH-terminal sequence (alanine 442–end including the proline-rich sequence), however, abolished the interaction (Fig. 3 B, pGADBA2). The deletion of the sequence for a.a. 436–464, including the proline-rich sequence, also abolished the interaction (Fig. 3 B, pGADAPRO). Proline-rich sequences are reported to be the motif of SH3 domain–binding sites (e.g., Cicchetti et al., 1992). These results suggest that the interaction between Boi2p and Bem1p is mediated by the interaction between the proline-rich sequence of Boi2p and SH3-2 of Bem1p. Deletion of the NH2-terminal sequence (a.a. 1–266), however, abolished the interaction (Fig. 3 B, pGAD2A2). It is likely that the NH2-terminal sequence affects the conformation of Boi2p, allowing the proline-rich sequence to bind efficiently or, alternatively, the NH2-terminal sequence might stabilize the Gal4–Boi2p fusion protein.

**The Interaction of Bem1p and Boi2p In Vitro**

The interaction of Boi2p with Bem1p was examined in vitro with purified GST-Boi2p. Yeast cells were transformed with the plasmids for myc-tagged Bem1p, shown diagrammatically in Fig. 1 A. The myc-tagged Bem1p in the lysates was detected with anti-myc antibodies (Fig. 3 C, left). The lysates containing myc-tagged Bem1p were incubated with GST-Boi2p that had been bound to GSH beads. The versions of Bem1p that possessed SH3-2 were coprecipitated with GST-Boi2p (Fig. 3 C, right, lanes 1, 2, and 5), whereas the versions of Bem1p that lacked SH3-2 only coprecipitated at very low levels, if at all, with GST-Boi2p (Fig. 3 C, right, lanes 3, 4, and 6). Both the two-hybrid experiments and the in vitro binding assays indicate that SH3-2 is critical for the Boi2p–Bem1p interaction and that SH3-1 is not essential for this interaction.

**Phenotypes of Cells that Overexpressed BOI1 and BOI2**

BOI1 was identified during a screening for genes whose expression, under the control of the GAL10 promoter, was lethal to yeast cells. The isolated BOI1 was fused to the GAL10 promoter at the Sau3AI site located in the sequence for codons 374–375 of BOI1. In this fused gene, the 378th methionine codon is expected to be the initiation codon. Cells that carried the multicopy plasmid that harbored the BOI1 sequence under the control of the GAL10 promoter did not grow on SCGal-L, a galactose-containing selective medium, and they were arrested as large,
Figure 2. Amino acid sequence of Boi2p. (A) The nucleotide and deduced amino acid sequences of BOI2. The nucleotide sequence of BOI2 has been deposited in DDBJ/EMBL/Genbank under the accession No. D38310. The Sau3AT restriction site, namely, the site of the junction with the sequence for Gal4 TA in pGADBOI2, is indicated by a box. The restriction sites for BstEII, XbaI, HindIII, BanII, AatII, EcoRI, and BglII, which were used for the construction of truncated versions of BOI2 (shown schematically in Fig. 3 B) are underlined. (B) Comparison of the amino acid sequences of Boi2p and Boilp. Identical residues are boxed in black. Domains I, II, III, and IV are underlined.
The overproduction of Boi2p without its NH2-terminal domain and in cells in which beml-ASH3s were introduced into IA0 cells that harbored either pGADBOI2 or pGAD424 (as a control), and proteins coprecipitated with GST-Boi2p beads (right panel, lanes 1–7) or GST beads (right panel, lane 8) as a control. Total cell lysates were resolved by SDS-PAGE, and myc-tagged Bemlp was detected by Western blotting analysis.

To identify the domain that is important for the inhibitory effect, we constructed two truncated versions of BOI2, namely pGAL7:BOI2ΔN and pGAL7:BOI2ΔC. The overproduction of Boi2p without its NH2-terminal half from pGAL7:BOI2ΔC, however, did not have any inhibitory effect on cell growth (Fig. 4 B, sector 2). These results indicate that the COOH-terminal half that contains domain IV is important for inhibition of bud emergence, while the NH2-terminal half that contains domains I, II, and III is not essential for the inhibition.

The finding that the NH2-terminal half is not essential for the inhibition suggests that the Bem1p–Boi2p interaction is not critical for the inhibitory effect. To clarify this, we introduced both constructs for the overexpression of BOI genes (pGA68 and pGAL7:BOI2) into bem1 mutant cells in which bem1 lacked the sequence for both SH3 domains (bem1-ΔSH3s). The overexpression of either BOI1 or BOI2 inhibited the growth of the bem1-ΔSH3s cells (data not shown). These results indicate that the protein–protein interaction between Bem1p and the Boi proteins is not critical for the inhibitory activity of the overexpressed BOI gene.
Phenotypes of the boi-disrupted Cells

Disruption of neither BOI1 nor BOI2 alone resulted in a cell growth defect (Fig. 5 A, sectors 2 and 3). However, all but one of ~200 Δboi1 Δboi2 spores isolated from Δboi1/Δboi2/+ heterozygous diploid cells failed to form colonies. The single viable Δboi1 Δboi2 spore formed a tiny colony that grew very poorly. Δboi1 Δboi2 cells with BOI2 under the control of the GAL7 promoter (pGAL7:BOI2) grew on medium that contained 0.1% galactose, which induced the expression of pGAL7:BOI2 with low efficiency. Δboi1 Δboi2 pGAL7:BOI2 cells dramatically reduced the growth rate 24 h after a shift to glucose-containing medium, which repressed the expression of pGAL7:BOI2 and then grew very poorly (Fig. 5 A, sector 4). Δboi1 Δboi2 pGAL7:BOI2 cells grew very poorly on glucose-containing medium at all temperatures tested (20, 25, 30, and 37°C). These results indicate that BOI1 and BOI2 are functionally redundant, and that the Boi proteins are important for cell growth.

About 24 h after the shift to glucose-containing medium, Δboi1 Δboi2 pGAL7:BOI2 cells began to stop growing and lyse, as judged from the leakage of alkaline phosphatase into the medium (Fig. 5 B). From the staining of...
dead cells with methylene blue (Rose, 1975), it appeared that ~30% of all the cells in the culture 24 h after the shift were dead, and ~95% of these dead cells were budded (Figs. 6 a). Within 48 h after the shift, the fraction of dead budded cells increased to ~60% of the total cells. The remaining cells were large and round (Fig. 6 a), and in these large round cells, the asymmetric organization of actin filament (as observed in wild-type cells; Fig. 6 b) was disrupted (Fig. 6 d). A majority of \( \Delta boi1 \Delta boi2 \) cells lysed with buds, and the death of these \( \Delta boi1 \Delta boi2 \) cells was not suppressed by the addition of an osmotic stabilizer into the medium (data not shown). In the presence of an osmotic stabilizer that partially prevented cell lysis, however, most of the \( \Delta boi1 \Delta boi2 \) cells were uniformly observed as large and round cells in the glucose-containing medium rather than as dead budded cells. Furthermore, the asymmetric organization of actin filaments was disrupted in these large round cells (Figs. 6, e and f), indicating that these \( \Delta boi1 \Delta boi2 \) cells have lost cell polarity. These morphological observations strongly suggest that cells without the Boi function failed to maintain cell polarity, and that the loss of cell polarity caused a defect in bud growth and subsequent cell lysis.

The Boi2p Domain That Is Required for Cell Growth

To identify the domain that is critical for the Boi function, we constructed two truncated versions of \( BOI2: \) \( BOI2\Delta N \), which lacked the sequence for domains I, II, and III; and \( BOI2\Delta C \), which lacked the sequence for domain IV. \( \Delta boi1 \Delta boi2 \) cells carrying \( BOI2\Delta N \) grew as well as wild-type cells (Fig. 5 A, sector 5), but \( \Delta boi1 \Delta boi2 \) cells carrying \( BOI2\Delta C \) on either a low copy number plasmid or a high copy number plasmid grew as poorly as \( \Delta boi1 \Delta boi2 \) cells (Fig. 5 A, sectors 6 and 7). These results indicate that the COOH-terminal half is essential and sufficient for cell growth while the NH2-terminal half is dispensable.

The Effect of BOI2 on \( \Delta rho3 \) Cells

Boi2p interacted with SH3-2, which was required for suppression by Bem1p of the \( \Delta rho3 \) defect. This result may suggest the involvement of Boi2p in the suppression. We examined whether or not multiple copies of \( BOI2 \) could suppress the \( \Delta rho3 \) defect. Although the overexpression of \( BOI2 \) from \( pGAL7:BOI2 \) inhibited cell growth, the introduction of \( pYO324-BOI2 \), a high copy number plasmid that harbored \( BOI2 \), into cells did not inhibit the growth of wild-type cells or \( \Delta rho3 \) cells that had been rescued by overexpression of \( RH04 \) (data not shown; Fig. 7, left). The growth of \( \Delta rho3 \) cells that carried \( pYO324-BOI2 \) was as poor as that of \( \Delta rho3 \) cells without \( pYO324-BOI2 \) (Fig. 7, right, sectors 1 and 2), indicating that \( BOI2 \) can not serve as a multicopy suppressor of the \( \Delta rho3 \) defect. Conversely, multiple copies of \( BOI2 \) inhibited the growth of \( \Delta rho3 \) cells that overexpressed \( BEM1 \). The growth defect of \( \Delta rho3 \) cells was rescued by overexpression of \( BEM1 \) (Fig. 7, right, sector 3), but in the presence of \( pYO324-BOI2 \), the growth of the \( \Delta rho3 \) cells that overexpressed \( BEM1 \) was as poor as that of \( \Delta rho3 \) cells without the \( BEM1 \) overexpression plasmid (Fig. 7, right, sector 4). However, neither \( BOI2\Delta N \) nor \( BOI2\Delta C \) on a high copy number plasmid did not show the inhibitory effect (Fig. 7, right, sectors...
5 and 6). These results indicate that the NH$_2$-terminal half and the COOH-terminal half of Boi2p is critical for the inhibitory activity. It might be possible that the BEM1-overexpressing Δrho3 cells are hypersensitive to the inhibitory effect of BOI2 on bud emergence, which was observed in case of pGAL7:BOI2. Such was not the case, however, since in addition to the difference in required domains, the BEM1-overexpressing Δrho3 cells that harbored pYO324-BOI2 showed morphology similar to that of Δrho3 cells without the BEM1 overexpression plasmid (showing an increase of the proportion of lysed cells with buds) instead of becoming large, round, unbudded cells (data not shown). These results suggest that Boi2p inhibits the function of Bem1p in the suppression of the Δrho3 defect.

**Genetic Interactions among the BOI Genes, RHO3, and RHO4**

Since (a) Boi2p interacts with Bem1p, (b) BEM1 interacts genetically with RHO3, and (c) the morphological pheno-
Figure 7. Effect of multiple copies of BOI2 on the growth of \( \Delta \)rho3 cells. \( \Delta \)rho3 pGAL7:RHO4 strain YMR505, carrying the indicated plasmids, was incubated on SCGal-UT (left) and SC-UT (right) plates at 30°C for 4 d. The \( \Delta \)rho3 strain YMR505 grew very poorly on glucose-containing medium but grew well on galactose-containing medium as a result of the overexpression of RHO4 from pGAL7:RHO4 (Matsui and Toh-e, 1992b). Plasmids were as follows: pYO324 and pKT10, dummy plasmids (sector 1); pYO324-BOI2, a high copy number plasmid that harbored BOI2, and pKT10 (sector 2); pBEM1KK, a BEM1-overexpressing plasmid, and pYO324 (sector 3), pBEM1KK and pYO324-BOI2 (sector 4); pBEM1KK and pYO324-BOI2AN, a high copy number plasmid that harbored BOI2AN (sector 5); pBEM1KK and pYO324-BOI2AC, a high copy number plasmid that harbored BOI2AC (sector 6).

Figure 8. Suppression of the boi defect. Wild-type cells (strain W303-1A, sector 1), \( \Delta \)boi1 \( \Delta \)boi2 pGAL7:BOI2 cells (strain YMR1207, sector 2), a \( \Delta \)boi1 \( \Delta \)boi2 segregant carrying pOPR3 (sector 3), and a \( \Delta \)boi1 \( \Delta \)boi2 segregant carrying pOPR4 (sector 4) were streaked on a YPD plate and incubated for 2 d at 30°C.

(type of the \( \Delta \)boi1 \( \Delta \)boi2 cells is similar to that of \( \Delta \)rho3 cells (Matsui and Toh-e, 1992b), we examined whether BEM1, RHO3, or RHO3-related genes could serve as multicopy suppressors of the boi defect. Plasmids were introduced into \( \Delta \)boi1/+ \( \Delta \)boi2/+ heterozygous diploid cells, and the transformants were sporulated and dissected. After incubation at 25°C for 3 d, \( \Delta \)boi1 \( \Delta \)boi2 segregants carrying plasmids that overexpressed either RHO3 or RHO4 (i.e., plasmids pOPR3 or pOPR4) formed visible colonies. They grew as well as wild-type cells and much better than \( \Delta \)boi1 \( \Delta \)boi2 cells without pOPR3 and pOPR4 (Fig. 8). These results indicate that both RHO3 and RHO4 can serve as a multicopy suppressor of boi. Plasmids overexpressing either BEM1 or RHO1, encoding a Rho-type GTPase, (i.e., pBEM1KK or pOPR1) did not rescue the growth defect of \( \Delta \)boi1 \( \Delta \)boi2 segregants (data not shown).

Discussion

Functions of the Boi Proteins

The phenotypes of \( \Delta \)boi1 \( \Delta \)boi2 cells resemble those of cells depleted of both Rho3p and Rho4p. \( \Delta \)rho3 \( \Delta \)rho4 cells carrying pGAL7:RHO4, RHO4 under the control of the GAL7 promoter cease to grow in a glucose-containing medium, and the Rho3p- and Rho4p-depleted cells lyse with buds (Matsui and Toh-e, 1992b; Fig. 5 B). In the presence of an osmotic stabilizer, Rho3p- and Rho4p-depleted cells become large and round, and the asymmetric organization of actin filaments is disrupted (Matsui and Toh-e, 1992b). The phenotypes of the Boi protein-depleted cells (Figs. 5 B and 6) were quite similar to those of Rho3p- and Rho4p-depleted cells, and they strongly suggest that these cells are defective in the maintenance of cell polarity for bud growth. Moreover, both RHO3 and RHO4 can serve as a multicopy suppressor of the boi defect. Both the strong genetic interactions and the phenotypes of mutants strongly suggest that the Boi proteins, Rho3p, and Rho4p are all involved in the same process that maintains cell polarity for bud growth.

Domains of Boi Proteins

Boi2p possesses four domains that are highly conserved in Boi1p (Fig. 2 B), and the high degree of conservation suggests that the domains play an important role in the function of the Boi proteins. Domain III contains a proline-
rich sequence that is required for displaying the two-hybrid interaction with SH3-2 of Bem1p (Fig. 3). Domain I contains an SH3 domain that can interact with an SH3 domain–binding protein. Domain IV contains a PH domain. We analyzed the role of the domains using truncated versions of Boi2p, and the results are summarized in Fig. 9. BOI2ΔN, which lacked the sequence for domains I-III, complemented Δboi1 Δboi2, while BOI2AC, which lacked domain IV, did not, an indication that the COOH-terminal half, including domain IV, of Boi2p is essential and sufficient for the function of Boi2p in cell growth, while the NH2-terminal half is dispensable. The role of a PH domain is still obscure, but it has been reported that PH domains participate in interactions with lipid moiety and proteins (Musacchio et al., 1993; Harlan et al., 1994). It is possible that Boi proteins interact with proteins other than Bem1p via the PH domain. In this context, it is of interest to recall that the overexpression of BOI genes under the control of the galactose-dependent promoter inhibited bud emergence (Fig. 4). As in the case of the function of Boi2p in cell growth, the COOH-terminal half of Boi2p (from pGAL7:BOI2ΔN) is essential and sufficient for the inhibitory effect, and the interaction of Bem1p and the Boi proteins is not required. These findings suggest that the COOH-terminal half of Boi2p interacts with a factor(s) involved in bud emergence. It might be possible that the interaction between the COOH-terminal half and the component(s) for bud emergence can replace, in part, the role of the Bem1p–Boi2p interaction and can allow cells to grow well without the NH2-terminal half of the Boi proteins.

The Role of the Bem1p–Boi2p Interaction

Although the NH2-terminal half of Boi2p is not essential for either cell growth or inhibition of bud emergence, the NH2-terminal half of Boi2p might be required to inhibit the suppression of the growth defect of Δrho3 cells by Bem1p. These findings strongly suggest that the Boi proteins possess the potential to modulate the function of Bem1p and that the NH2- and COOH-terminal portions of the Boi proteins play a critical role in modulating the function of Bem1p. The NH2-terminal portion of Boi2p interacts with SH3-2 of Bem1p, and SH3-2 is critical for suppression of the Δrho3 defect. Thus, it is likely that the Bem1p–Boi2p interaction plays a role in the modulating activity.

The role of the Bem1p–Boi2p interaction in the suppression of the Δrho3 defect is suggested to be negative; multiple copies of BOI2 did not suppress the Δrho3 defect, but they inhibited the growth of Δrho3 cells that had been rescued by the overproduction of Bem1p (Fig. 7). From these results, we cannot exclude the possibility that the intrinsic amount of Boi2p might play a positive role in the suppression of the Δrho3 defect. It is possible, however, that another protein that interacts with SH3-2 might play an important role in suppressing the Δrho3 defect, acting in concert with or independently of Boi2p, and that Boi2p might modulate the function of Bem1p by interacting competitively with SH3-2. It was reported very recently that Bem1p interacts with Ste20p and Ste5p, two components of the pheromone-responsive mitogen-activated protein kinase cascade, as well as with actin. The Ste20p protein kinase requires both SH3-2 and the COOH-terminal half of Bem1p for the Ste20p–Bem1p interaction (Leeuw et al., 1995). Ste20p is therefore a candidate for a protein that is critical for the suppression of the Δrho3 defect. At this time, however, we have no experimental evidence to suggest the involvement of Ste20p.

Protein Complex for Bud Formation

The COOH-terminal 35 a.a. of Bem1p is required for bud emergence at elevated temperatures and for the suppression of the rho3 defect (Fig. 1). Thus, it appears possible that protein–protein interaction at the COOH terminus of Bem1p is critical for the functions of Bem1p. One of the candidates for an interacting protein is Cdc24p (Peterson et al., 1994). Indeed, the COOH-terminal 35 a.a. was required for the two-hybrid interaction between Bem1p and Cdc24p (Table II). Cdc24p also interacts with Cdc42p as a GTP–GDP exchange factor and with Rsr1p/Bud1p, which is needed for determination of the bud site (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Zheng et al., 1994, 1995). The finding that Bem1p required the Cdc24p–interacting COOH terminus for suppression of the Δrho3 defect suggests the possibility that a protein complex containing Bem1p and Cdc24p might play a role in the process that involves Rho3p. It is plausible that the protein complex for bud-site selection is developed by the association of Cdc42p, Cdc24p, and Bem1p for the polarization of cells. After the initiation of bud emergence, the protein complex for cell polarization should be developed and/or rearranged to terminate the process of initiation of bud emergence and for the continuation of bud growth. In this context, it is of great interest that factors involved in bud growth exhibit genetic and protein–protein interactions with factors that are involved in bud emergence and, moreover, that Boi2p might be able to modulate the function of Bem1p and to interact with a factor(s) other than Bem1p that is involved in cell polarization. Our present working hypothesis is that for the regulation of cell mor-

![Figure 9](image-url) Summary of the domain analysis of BOI2. The BOI2 sequence of each allele is indicated by a thick line below the coding region of BOI2 (open box). The SH3 domain (hatched), the proline-rich sequence (black), and the PH domain (cross-hatched) are indicated. The regions of domains I, II, III, and IV are indicated by narrow lines. The abilities of each allele to complement Δboi1 Δboi2, to inhibit bud emergence of wild-type cells when overexpressed under the control of the GAL7 promoter, and to inhibit the growth of the BEM1-overexpressing Δrho3 cells are indicated.
phogenesis, Rho3p and Boi proteins control the development of the protein complex for bud growth, and the Boi proteins mediate the connections between the protein complex for cell polarization and the machinery for bud growth via its affinities for Bem1p and for the factor(s) involved in cell polarization.

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